Ah receptor activation impairs cortisol response to stress in rainbow trout by disrupting the rate limiting steps in steroidogenesis

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Abstract

Anthropogenic stressors activating aryl hydrocarbon (Ah) receptor signaling, including polychlorinated biphenyls, impair the adaptive corticosteroid response to stress, but the mechanisms involved are far from clear. Using Ah receptor agonist (β-naphthoflavone; BNF) and antagonist (resveratrol; RVT), we tested the hypothesis that steroidogenic pathway is a target for endocrine disruption by xenobiotics activating Ah receptor signaling. Trout (*Oncorhynchus mykiss*) were fed BNF (10 mg/kg/day), RVT (20 mg/kg/day) or a combination of both (RBNF) for 5 days, and subjected to a handling stress. BNF induced cytochrome P4501A1 (CYP1A1) expression in the interrenal tissue and liver, while this response was abolished by RVT, confirming Ah receptor activation. In control fish, handling stress transiently elevated plasma cortisol and glucose levels and transcript levels of interrenal steroidogenic acute regulatory protein (StAR), cytochrome P450 cholesterol side chain cleavage (P450scc) and 11β-hydroxylase over a 24 h period. BNF treatment attenuated this stress-induced plasma and interrenal responses; these BNF-mediated responses were reverted back to the control levels in the presence of RVT. We further examined whether these in vivo impact of BNF on steroidogenesis can be mimicked in vitro using interrenal tissue preparations. BNF depressed ACTH-mediated cortisol production and this decrease corresponded with lower StAR and P450scc, but not 11β-hydroxylase mRNA abundance. RVT eliminated this BNF-mediated depression of interrenal corticosteroidogenesis in vitro. Altogether, xenobiotics activating Ah receptor signaling are corticosteroid disruptors, and the mode of action includes inhibition of StAR and P450scc, the rate limiting steps in steroidogenesis.

Introduction

Corticosteroids, synthesized by the adrenal cortex, are involved in the regulation of numerous physiological processes including intermediary metabolism, ion regulation and immune responses (1, 2). Their secretion is tightly regulated by hormones released from the hypothalamus and pituitary, including corticosteroid -releasing hormone (CRH) and adrenocorticotropic hormone (ACTH), respectively. As in mammals, corticosteroid biosynthesis and secretion in teleost fishes are also under the control of the hypothalamus-pituitary axis, but unlike mammals they lack a discrete adrenal gland. Instead, the corticosteroidogenic cells (interrenal cells) are distributed around the post-cardinal vein in the anterior part of the kidney and secrete cortisol, the primary corticosteroid in bony fishes, in response to stressors (3, 4).

Cortisol biosynthesis involves a series of steps commencing with the stimulation of interrenal tissues by ACTH and the subsequent conversion of cholesterol, through a series of enzymatic steps, including cytochrome P450 family proteins, dehydrogenases and hydroxylases, to steroids (5). Steroid acute regulatory protein (StAR), a rate-limiting step in steroidogenesis, plays a key role in the transport of cholesterol from outer to inner mitochondrial membrane (6). This provides the substrate for the primary rate-limiting step in the steroidogenic pathway, catalyzed by cytochrome P450 side chain cleavage (P450scc), converting cholesterol to pregnenolone. Cortisol is synthesized from pregnenolone through a series of isomerizations and hydroxylations, including the final step catalyzed by 11β-hydroxylase (5, 7). Key players in the corticosteroidogenic response to stress includes StAR and P450scc, both acutely regulated by trophic hormones (8, 9, 10, 11, 12), while enzymes downstream of P450scc, including 11β-hydroxylase, retain the steroidogenic activity even in the absence of trophic hormone stimulation (13, 14).

Elevation of circulating cortisol levels in response to stressors is considered an adaptive response and important for homeostatic adjustments to cope with stress (1, 2). This is also true in teleost fishes (3, 4), but persistent organic pollutants, including polychlorinated biphenyls (PCBs), impair this adaptive response by decreasing the interrenal capacity for cortisol production (16). Recent studies have proposed that PCBs act at multiple sites along the hypothalamus-pituitary-interrenal axis to weaken the stressor-mediated cortisol response, including decreased sensitivity of interrenals to trophic hormones (15, 16, 17), abnormal negative feedback regulation (18), altered cortisol clearance (4) and inhibition of steroidogenesis (19).

Indeed, the resistance to 2,3,7,8-tetracholorodibenzo-p-dioxin (TCDD)-induced toxicity in Ah receptor null mice, including lack of changes in steroidogenic enzymes, suggests a link between Ah receptor signaling and impaired steroidogenesis (20, 21, 22). However, the mode of action of xenobiotics, acting via Ah receptor signaling, in impairing corticosteroid response to stress is far from clear. Against this backdrop, we tested the hypothesis that the rate limiting steps in steroidogenesis are key targets for endocrine disruptors activating Ah receptor signaling in rainbow trout, and leads to impaired cortisol response to stress. Fish were administered Ah receptor agonist (β-naphthoflavone, BNF) and antagonist (resveratrol, RVT; 23,24) either alone or in combination to tease out the role of Ah receptor activation on plasma cortisol response and interrenal corticosteroid biosynthesis.

Materials and Methods

Chemicals

β –Naphthoflavone, resveratrol (99 % purity), protease inhibitor cocktail, bicinchoninic acid (BCA) reagent, L15 medium, porcine ACTH $_{(1\text{-}39)}$ and 2-phenoxyethanol were obtained from Sigma-Aldrich (St. Louis, MO, USA). All electrophoresis reagents and molecular weight markers were from BioRad. CYP1A1 antibody (mouse anti-cod CYP1A1 monoclonal antibody) was from Biosense laboratories (Bergen, Norway) and the secondary antibody, alkaline phosphatase-conjugated goat anti-mouse IgG, was from Sigma. Nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (BCIP) were from Fisher Scientific (Ontario).

Fish

Juvenile rainbow trout (*Oncorhynchus mykiss*, average body mass 227 g) were obtained from Humber Springs trout hatchery, Mono Mills, Ontario, Canada. Fish were acclimated in 2000 liter tanks with continuous running water at 13^{o}C and 12L: 12D photoperiod for 3 weeks before the experiment. The fish were fed to satiety (3 point sinking food, Martin Mills Inc., Elmira, Ontario, Canada) once daily 5 days a week.

Protocols

Role of Ah receptor in the stress response

Groups of 8 fish each were randomly distributed in 12 tanks (100 liter) and acclimated for two weeks prior to the experiment. Treatments were assigned to the tanks (3 tanks per treatment x

4 treatments) randomly and the fish were fed with feed laced with either vehicle (ethanol laced feed) or vehicle containing resveratrol (RVT; 20 mg/kg body mass/day), β -naphthoflavone (BNF; 10 mg/kg body mass/day) or a combination of BNF and RVT (RBNF; 20 mg RVT and 10 mg BNF/kg body mass/day) for 5 days. Briefly, the feed was laced by evenly submerging in 95% ethanol alone (control) or ethanol containing RVT, BNF or RBNF to ensure adequate coating of the pellets. The ethanol was allowed to evaporate by air-drying and the feed was stored in a cool and dry place. This method provides an easy means of administering the drugs without stressing the fish unlike intraperitoneal injections or implants (25).

After 5 days of feeding, 6 fish from each treatment (2 fish from each tank) were sampled quickly and killed with an overdose of 2-phenoxyethanol (1:1000) and these were the unstressed (0 h) control fish. The remaining fish were subjected to a standardized stress protocol of netting and chasing the fish for 5 min and were left undisturbed until they were sampled at 1, 4 and 24 h post-stress. Also, an additional 4 tanks (5 fish per tank) were maintained exactly as mentioned above as unstressed controls and sampled at 0, 1, 4 and 24 h to take into consideration the effect of sampling time on the measured parameters. Sampling consisted of quickly netting all fish from each tank, bleeding by caudal puncture into heparinized tubes, and the plasma was collected after centrifugation (6000 X *g* for 10 min) and stored frozen (-70^{o}C) for plasma cortisol and glucose determination. Pieces of liver and head kidney were frozen immediately on dry ice for protein and mRNA determinations respectively (see below).

Temporal profile of StAR, P450scc and 11β-hydroxylase in head kidney

Time course profiles of ACTH-stimulated increase in StAR, P450scc and 11β-hydroxylase mRNA abundance were determined by in vitro incubation of head kidney slices following previously standardized procedure (19). Briefly, fish were killed with an overdose of 2-phenoxyethanol (1:1000) and head kidney tissue was excised immediately and sliced into 1mm^3 pieces in ice-cold L15 media. The head kidney slices were distributed equally into 12 wells (six each for control and ACTH) in 24 well tissue culture plate (Corning Inc., Corning, NY). Tissue pieces were allowed to incubate with gentle shaking for 2 h at 13^{o}C (equilibration period), after which the L15 medium was replaced with fresh medium containing either no ACTH (control wells) or ACTH (0.5 IU/ml; ACTH wells) and incubated for 0, 0.5, 1, 2, 3 and 4 h. The tissue slices were collected at the end of each time point and frozen for later determination of StAR, P450scc and 11β-hydroxylase mRNA levels. For each experiment, head kidney tissue from 2-3

fish were pooled and this was repeated at least four times using fresh head kidney tissues from different fish (n = 4). The concentration of ACTH was determined previously by dose-response studies and 0.5 IU/ml ACTH elicited maximal cortisol production in trout head kidney tissues (17).

Role of Ah receptor on steroidogenesis in vitro

The role of Ah receptor on acute regulation of interrenal steroidogenesis was assessed by in vitro exposure of head kidney tissue to BNF and RVT either alone or in combination and determining the steroidogenic capacity in response to ACTH stimulation. The incubation consisted of slicing and distributing the tissues (as described above) from each fish and equally splitting them into 8 wells (two wells (one each for control and ACTH) per treatment x 4 treatments). The tissue pieces were allowed to incubate with gentle shaking for 2 h at 13^{o}C (equilibration period), after which the supernatant was replaced with fresh medium and treated with either control (0.01% DMSO) or RVT (10^{-5} M), BNF (10^{-6} M) and RBNF (10^{-5} M RVT and 10^{-6} M BNF). In RBNF group, RVT was added 30 min prior to the addition of BNF. The tissues were incubated for 1 h and the media collected for later determination of cortisol concentration (basal cortisol production rate). The media was replaced with fresh media along with treatments and were stimulated either with or without ACTH (0.5 IU/ml) for 3 h. After 3 h the supernatant was collected and frozen for later determination of cortisol concentration (stimulated cortisol production rate). Wet mass of the tissue in each well was recorded and cortisol production rate was expressed as ng/h/mg wet weight. The head kidney tissue was frozen for later determination of steroidogenic enzyme mRNA abundance. This was repeated at least four times with fresh head kidney tissue from different fish.

Cortisol and glucose

Plasma cortisol concentrations were measured using a commercially available ImmuChem$^{TM\ 125}$I RIA kit (ICN biomedicals, CA) according to established protocols (17). Plasma glucose levels were determined colorimetrically (Trinder method; Sigma).

SDS-PAGE and Western blotting

Protein concentrations were determined using the bicinchoninic acid method with bovine serum albumin as the standard. The procedure for SDS-PAGE and western blotting were according to established protocols (17). Briefly, samples (40µg protein /sample) were separated on 8% polyacrylamide gels using a discontinuous buffer system (26). The proteins were

transferred onto a nitrocellulose membrane (20 V for 20 min) with a semidry transfer unit (BioRad) using transfer buffer (25mM Tris pH 8.3, 192 mM glycine, and 20 % (v/v) methanol). The membrane was blocked with 5 % skimmed milk in TBS-t (20mM Tris pH 7.5, 300mM NaCl and 0.1% (v/v) Tween 20 with 0.02 % sodium azide) for 60 min. The mouse monoclonal anti-cod CYP1A1 primary antibody (1:3000) and alkaline phosphatase-conjugated goat anti-mouse secondary antibody (1:1000 dilution) were diluted in the blocking solution. The membranes were incubated in primary antibody for 60 min at room temperature, washed with TBS-t (2 × 5 min), incubated with secondary antibody for 60 min, washed with TBS-t (2 × 5 min), and finally washed with TBS (1 × 15 min). Visualization of bands was carried out with NBT (0.033 % w/v) and BCIP (0.017 % w/v) and the molecular mass confirmed using prestained low molecular weight marker (BioRad). Quantification of bands was carried out with Chemi imager^{TM} using the AlphaEase software (Alpha Innotech, CA).

Quantitative real-time PCR

RNA isolation and first strand cDNA synthesis

Total RNA was isolated from head kidney using RNeasy mini-kit (Qiagen, ON) following the manufacturer's protocol and quantified spectrophotometrically at 260 nm. RNA was DNase treated to avoid genomic contamination following manufacturer's instructions. The first strand cDNA was synthesized from 1 μg of total RNA using First Strand cDNA synthesis kit (MBI Fermentas). Briefly, total RNA was heat denatured (70°C) and cooled on ice. The sample was used in a 20 μl reverse transcriptase reaction using 0.5 μg oligo d(T) primers and 1mM each dNTP, 20 U ribonuclease inhibitors, and 40 U M-MuLV reverse transcriptase. The reaction was incubated at 37°C for 1 h and stopped by heating at 70 °C for 10 min.

Relative standard curve

Primers were designed using rainbow trout CYP1A1, StAR, P450scc, 11β-hydroxylase and β-actin cDNA sequences and the size of the amplified product was 100 bp for CYP1A and β-actin and 500 bp for StAR, P450scc and 11β –hydroxylase (Table 1). A relative standard curve was constructed for target genes (CYP1A1, StAR, P450scc and 11β-hydroxylase) and house-keeping gene (β-actin) using either cDNA stock or plasmid vectors with inserted target sequences according to established protocols (27). Briefly, the concentration of cDNA or plasmid vector stock was assumed to be 500 pg μl^{-1} and reactions were setup with different concentrations ranging from 10 to 3000 pg per reaction for the standard curve (27). Platinum® Quantitative PCR

SuperMix-UDG (Invitrogen, CA) used was 2X concentrated and every 25 µl reaction had 1.5 U Platinum Taq DNA polymerase, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 3 mM MgCl2, 200 µM dGTP, 200 µM dATP, 200 µM dCTP, 400 µM dUTP, and 1 U UDG; the reaction also contained 0.2 µM forward and reverse primers and 1:100,000 SYBR green I nucleic acid gel stain (Roche). Master mixes, to reduce pipetting errors, were prepared at every stage for triplicate reactions (3 x 25 µl) for each standard.

Quantification

One micro liter of cDNA sample was used as a template for every 25 µl reaction of target genes and half a micro liter of cDNA was used as a template for β-actin. Each sample was setup in triplicates for the qPCR. The reaction components were exactly the same as the previous section and for every single test sample, a qPCR for both the target (CYP1A1, StAR, P450scc and 11β-hydroxylase) and the housekeeping gene (β-actin) was performed. The following PCR cycle was used for gene amplification: 95 °C – 3 min; 40 cycles: denaturing 95 °C – 30 sec, annealing (Table 1) – 30 sec, extend 72 °C – 30 sec, followed by 4 °C hold at the end of the PCR reaction. The transcript abundance was obtained from their respective standard curves after normalization with β-actin; threshold cycles for β-actin showed little change with treatments and used for the normalization.

Statistical Analyses

Data were expressed as mean ± standard error of mean (S.E.M.) and log-transformed, wherever necessary, for homogeneity of variance, but non-transformed values are shown in the figures. Two-way analysis of variance (ANOVA) was used to determine the significant time, treatment and interaction effects on plasma cortisol and glucose concentrations. One-way ANOVA was used for all other comparisons, except a paired Student's t-test was used to compare the significant differences in steroidogenic enzyme mRNA levels between in vitro ACTH-stimulated and unstimulated groups. A posthoc (Bonferroni test) test was used for pair-wise comparison wherever significant differences were observed. A probability level of $p \leq 0.05$ was considered significant. All statistical analyses were performed with SPSS version 12.0.1 (SPSS Inc., Chicago, IL, USA).

Results

Ah receptor activation in vivo

CYP1A1 expression in vivo

BNF significantly elevated liver CYP1A1 protein expression compared to all other treatments (Fig. 1A). This BNF-induced CYP1A1 response was abolished by RVT (Fig. 1A). In head kidney, CYP1A1 protein expression was undetectable and, therefore, CYP1A1 mRNA abundance was ascertained by qPCR. As expected, CYP1A1 mRNA abundance was significantly higher with BNF compared to other treatments and this response was also eliminated by RVT (Fig. 1B).

Cortisol and glucose

In the unstressed control fish, plasma cortisol levels did not change with time in the present study (Fig. 2A). Handling disturbance significantly increased plasma cortisol levels at 1 h post-stress, but the levels declined thereafter and was not statistically different from the unstressed levels at 4 and 24 h post-stress (Fig. 2A). However, BNF but not other treatments significantly attenuated this stress-induced plasma cortisol elevation, while RVT (RBNF group) reverted this response back to the control levels (Fig. 2A). The pre-stress plasma cortisol levels were not significantly different between treatments.

Plasma glucose concentration also showed no significant time effect in the control unstressed fish (Fig . 2B). There was a significant time and treatment effects on plasma glucose levels after stressor exposure (Fig. 2B). Glucose levels peaked at 4 h post-stress and at 24 h the levels in all treatments were back to resting values. As seen with cortisol, BNF treatment attenuated the stress-induced elevation of plasma glucose concentration, while this response was offset by RVT (Fig. 2B). There were no treatment effects on pre-stress plasma glucose levels in the present study.

StAR, P450scc and 11β –hydroxylase

There was no significant effect of sampling time on StAR, P450scc and 11β-hydroxylase mRNA abundance in the unstressed groups (Figs. 3A-C). StAR and 11β –hydroxylase transcripts were significantly higher in response to handling stress at 1 h post-stress and the levels remained significantly elevated even at 4 and 24 h post-stress compared to unstressed controls (Figs. 3A and 3C). P450scc mRNA levels increased with handling stress with significantly higher levels observed at 4 h post-stress, but the levels returned to unstressed levels at 24 h post-stress (Fig. 3B).

No significant differences in the mRNA levels of StAR, P450scc and 11β-hydroxylase among treatments (control, BNF, RVT, RBNF) were observed prior to stress. However, BNF significantly attenuated both StAR and P450scc mRNA abundance at 1 h post-stress compared to all other groups (Figs. 4A and 4B). This BNF-mediated steroidogenic response was eliminated in the presence of RVT (RBNF group) in the present study. No significant difference in the mRNA abundance of 11β-hydroxylase was observed between treatments (Fig. 4C).

Ah receptor activation in vitro

CYP1A1 expression

In vitro administration of BNF to head kidney slices significantly elevated CYP1A1 mRNA abundance compared to other treatments. This BNF-induced elevation in the mRNA levels was blocked by RVT (RBNF group; Fig. 5A).

Cortisol production

BNF significantly attenuated *in vitro* ACTH-stimulated cortisol production compared to all other treatments (Fig. 5B). Administration of RVT prior to the addition of BNF overcame the BNF-mediated attenuation of ACTH-stimulated cortisol production (Fig. 5B). RVT alone did not significantly affect cortisol production.

StAR, P450scc and 11β-hydroxylase

Unstimulated head kidney tissue (sham) showed no significant changes in the mRNA abundance of StAR, P450scc and 11β-hydroxylase with sampling time in vitro (Figs. 6A–6C). In vitro stimulation of head kidney tissue with ACTH temporally elevated all three transcript levels (Fig. 6). StAR mRNA abundance was significantly higher at 0.5 h and stayed elevated over the 4 h time period (Fig. 6A). P450scc mRNA levels were significantly higher at 1 h post-ACTH stimulation and stayed elevated for the remaining sampling times (Fig. 6B). 11β-hydroxylase mRNA abundance also showed a significant elevation temporally, similar to that of P450scc; significantly higher at 1 h and remaining elevated over the 4 h period (Fig. 6C).

BNF administration significantly depressed ACTH-mediated increase in StAR and P450scc mRNA levels, but not 11β-hydroxylase mRNA abundance (Figs. 7A-7C). Also, RVT blocked this BNF-mediated decrease in StAR and P450scc mRNA abundance in response to ACTH stimulation (Figs. 7A and 7B). There was no effect of RVT either alone or in combination with BNF on 11β-hydroxylase mRNA levels (Fig. 7C).

Discussion

Ah receptor activation impairs cortisol response to stress in rainbow trout. Specifically, attenuation of StAR and P450scc mRNA levels by BNF corresponded with a reduction in the stress-induced cortisol response in vivo as well as cortisol production to ACTH stimulation in vitro. Resveratrol, a recently characterized and effective AhR antagonist (23,24) even in trout (N. Aluru and M.M. Vijayan, Submitted), abolished this BNF-mediated response providing the first clear evidence that Ah receptor activation targets the rate-limiting steps in steroid biosynthesis, resulting in the attenuation of the adaptive cortisol response crucial for homeostatic adjustments to stress.

Stress and corticosteroidogenesis

The initial step in corticosteroidogenesis involves the translocation of cholesterol from outer to the inner mitochondrial membrane by StAR, where it is converted to pregnenolone by P450scc (6). Both StAR protein and transcript levels are acutely regulated by trophic hormones in higher vertebrates (9, 28, 29, 30) and fish species (10, 11, 12). Our results are consistent with mammalian studies showing acute ACTH-stimulated increase in StAR mRNA levels within 30 min in interrenal cells in vitro. Also, in vivo handling stress elevated StAR mRNA levels at 1 h and remained elevated over the 24 h period post-stress. This prolonged elevation of the transcripts in vivo, even after plasma cortisol levels dropped to unstressed values, suggests increased mRNA stability, but if this has any functional significance remains to be elucidated. Further characterization of transcriptional, post-transcriptional (9), translational (31) and/or post-translational regulation (29, 32) of StAR expression in lower vertebrates, as demonstrated in mammalian models, can shed some light on the mechanism of regulation of this important step in steroidogenesis.

Similar to the acute regulation of StAR, steroidogenic enzymes (P450scc and 11β-hydroxylase) were also elevated with handling stress and ACTH stimulation. Upon handling stress, P450scc mRNA transcripts achieved peak levels by 4 h, and at 24 h post-stress the levels were similar to pre-stress levels, closely reflecting the plasma cortisol levels to stress in vivo. Also, in vitro ACTH stimulated P450scc transcript levels in head kidney slices over a 4 h period suggesting that the stress-induced elevation of this rate-limiting step in steroidogenesis may correspond to the transient plasma ACTH response seen with stress in fish (33). These results are in agreement with mammalian models clearly showing an elevation in P450scc transcript levels

in response to ACTH stimulation (29, 30). While P450scc transcript profile reflected more closely plasma cortisol levels post-stress in trout, 11 β-hydroxylase transcript levels remained elevated over the entire 24 h period after stress. This is in agreement with mammalian studies showing an upregulation of 11 β-hydroxylase transcript levels after ACTH treatment (30), leading to the suggestion that this enzyme is not acutely regulated by stress. Considered together, handling disturbance and the associated cortisol response involve upregulation of key steroidogenic enzymes in the interrenal tissue of rainbow trout. This concurs with a recent study that also showed an upregulation of StAR and P450scc transcripts in response to stress in rainbow trout (12). Given the cumulative plasma cortisol response to multiple stressors in salmonids (34), it is reasonable to propose that the elevated StAR and steroidogenic enzymes transcript level in response to initial stressor exposure may have adaptive significance for the rapid cortisol production in response to subsequent (multiple) stressor insults.

Ah receptor impact on corticosteroidogenesis

Several studies reported the attenuation of interrenal steroidogenic capacity by Ah receptor agonists, including decreased plasma cortisol response to stress and insensitivity to ACTH stimulation in both feral and hatchery reared teleost fishes (15). Our results are in agreement with those studies clearly showing an attenuation of either stress- or ACTH-induced cortisol production by BNF in rainbow trout (Fig. 1A and 6B). We did report a decrease in corticosteroid production with BNF recently and that corresponded with a depressed StAR and P450scc, but not 11β-hydroxylase mRNA abundance clearly establishing a mechanistic link for steroid inhibition (19). As this BNF-mediated depression of steroidogenesis was completely abolished with RVT, our results unequivocally demonstrate that Ah receptor signaling is involved in suppressing the rate limiting steps in steroidogenesis.

In higher vertebrates, although StAR was impacted by xenobiotics (35, 36, 37, 38), a clear role for Ah receptor signaling in regulating StAR gene expression was lacking (22). Indeed studies have shown an attenuation of trophic hormone-induced P450scc activity/expression with TCDD (39, 40, 41, 42). Also, recently using AhR-knockout mice, it was shown that TCDD impact steroidogenesis by affecting P450scc gene expression, but not StAR (22). Consequently, while Ah receptor signaling impacts P450scc in mammalian models, little is known about the role of this receptor on StAR regulation. Our study, both in vivo and in vitro, for the first time

clearly establishes StAR as a target for endocrine disruption by xenobiotics acting via Ah receptor (Fig. 4A and 7A). The lack of any impact on the 11β-hydroxylase mRNA accumulation by Ah receptor agonist in the present study also agrees with mammalian studies and this could possibly be due to upstream regulation of StAR and P450scc by Ah receptor (39). It is clear from these studies that the attenuated cortisol response to stress seen in fish in vivo with Ah receptor agonists (15, 16, 43) involves impairment of the rate limiting steps in interrenal steroidogenesis.

The mechanism(s) involved in the AhR-mediated attenuation of StAR and P450scc is not known, but few studies have attributed a negative interaction of AhR with the xenobiotic response elements (XREs) present on the steroidogenic enzymes as a possible mechanism of action (41). To our knowledge, only one steroidogenic enzyme (*cyp*19) promoter region was shown to have a XRE (44), but the possibility exists that XREs are present on the promoters of StAR and P450scc based on the responses observed with AhR agonists (22; this study). In addition to direct interaction, several other indirect mechanisms of interaction of AhR with steroidogenic enzymes have also been proposed. For instance, AhR competes with steroidogenic enzyme regulatory factors like orphan nuclear transcription factor SF-1, cAMP response element binding protein (CREBP) and CCAAT/enhancer binding protein (C/EBP) for co-activators and repressors like steroid receptor co-activator-1 (SRC-1) and Sp1, thereby modulating the genes involved in steroidogenesis (45, 46), but these interactions remains to be elucidated in fish. Taken together, it seems likely that the mode of action of AhR in impairing steroidogenesis is similar in all vertebrates. This is not surprising given the fact that the genes encoding AhR as well as the steroidogenic enzymes are highly conserved among vertebrates (47).

In conclusion, our results for the first time clearly define a role for Ah receptor in mediating the negative effects of PCBs on corticosteroidogenesis in vertebrates. Our result unequivocally demonstrate Ah receptor-mediated inhibition of StAR and P450scc gene expression in fish and suggests a mechanistic link for the impairment of cortisol response seen in teleost fishes from polluted waters, including PCB contaminated sites (15). As the rate limiting steps in steroidogenesis are not just limited to corticosteroids, but also reproductive steroids, our results emphasize that endocrine disruption with Ah receptor activation may impact all aspects of animal performance, including growth and metabolism, iono and osmoregulation, immune function and reproduction in fish. Consequently, xenobiotics activating Ah receptor signaling are more likely to be “homeostatic disruptors” in animals.

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Figure Legends

Figure 1. Liver CYP1A1 protein expression (A) and head kidney CYP1A1 mRNA abundance (B) in rainbow trout. Fish were given control feed or feed containing resveratrol (RVT; 20 mg/kg body mass/day), β-naphthoflavone (BNF; 10 mg/kg body mass/day) or RVT and BNF together (RBNF; 10 mg BNF/kg body mass/day and 20 mg RVT/kg body mass/day) for 5 days. A representative western blot and the corresponding histogram for the band intensities are shown for liver CYP1A1 protein expression. Samples are loaded in duplicate in adjacent lanes and are aligned to correspond with the treatments in the histogram. The samples were immunodetected for CYP1A1 protein using mouse monoclonal anti-cod antibody. All values represent mean + S.E.M. (n = 5- 6 fish); bars with different letters are statistically significant (One-way ANOVA, $p<0.05$).

Figure 2. Handling stress induced elevation in plasma cortisol and glucose concentrations in rainbow trout. Plasma cortisol (A) and plasma glucose (B) concentrations in fish fed either control feed or feed containing RVT, BNF or RBNF for 5 d and subjected to a standardized handling stress (5 min repeated netting and chasing). Plasma samples were collected at 0 (pre-stress), 1, 4 and 24 h post-handling stress. See figure 1 legend for details. All values represent mean ± S.E.M. (n = 5-6 fish); time points with different letters are statistically significant and the inset shows significant treatment effects (Two-way ANOVA, $p<0.05$). The unstressed group, for both cortisol and glucose, is shown just for reference and there was no statistical difference among the time points in that group.

Figure 3. Handling stress-induced changes in StAR (A), P450scc (B) and 11β -hydroxylase transcript levels in trout head kidney. Fish were either unstressed or stressed and sampled at 0, 1, 4 and 24 h later and transcript levels determined using real-time quantitative PCR; values are shown as percent pre-stress (0 h); all values represent mean ± S.E.M. (n = 6 fish for stressed group and n = 4 for unstressed group); different letters denote significant differences between time points (One-way ANOVA, $p<0.05$).

Figure 4. Ah receptor-mediated effects on StAR (A), P450scc (B) and 11β-hydroxylase (C) transcript levels at 1 h post-handling stress. Magnitude of change in mRNA levels was calculated

by deducting the pre-stress (0 h) levels from 1 h post-stress levels and expressed as percent control; transcript levels were measured using real-time quantitative PCR; all values represent mean + S.E.M (n = 4 fish); bars with different letters are statistically significant (One-way ANOVA, $p<0.05$).

Figure 5. Ah receptor activation impairs in vitro ACTH-stimulated corticosteroidogenesis in trout. CYP1A1 mRNA abundance (A) and ACTH-stimulated cortisol production (B) in head kidney tissue slices exposed to control (0.01% DMSO), resveratrol (10^{-5} M RVT), β-naphthoflavone (10^{-6} M BNF) or RVT and BNF (RBNF) before stimulation with ACTH (0.5 IU/ml). The magnitude of change in cortisol production was calculated by deducting the basal cortisol production from ACTH-stimulated increase and expressed as percent control; bars with different letters are statistically significant (One-way ANOVA, $p< 0.05$); all values represent mean + S.E.M. (n = 4 fish).

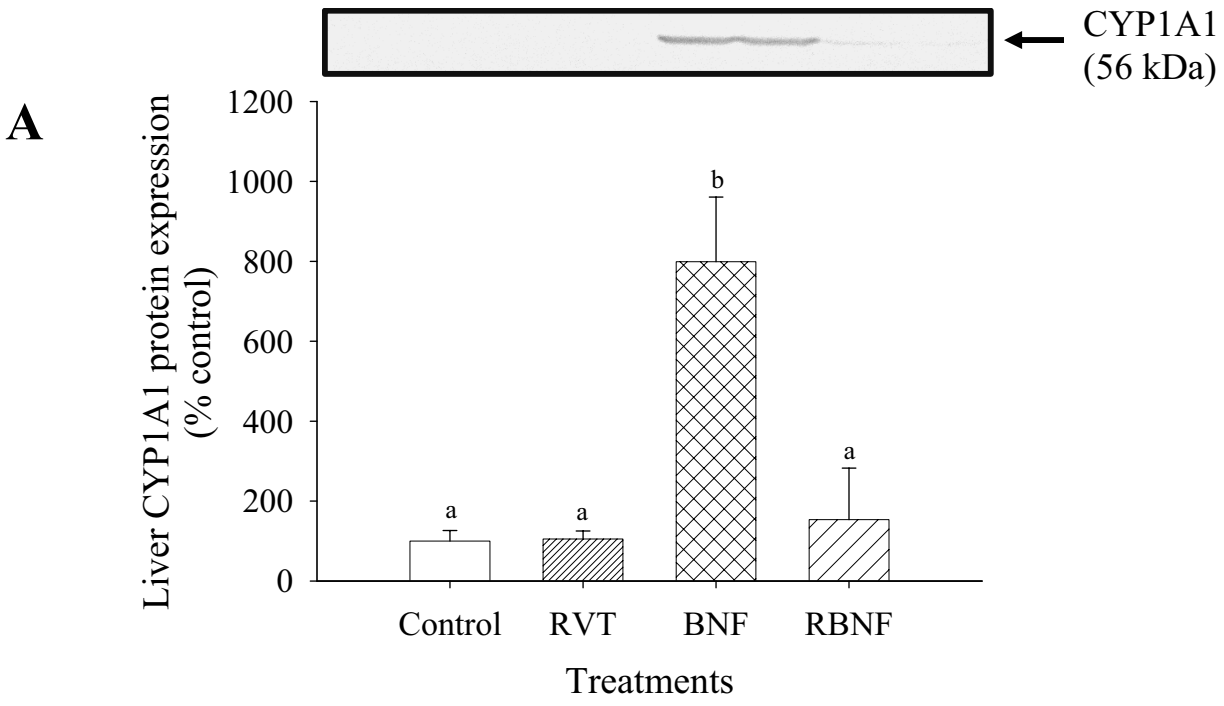
Figure 6. Temporal profiles of StAR (A), P450scc (B) and 11β-hydroxylase (C) mRNA abundance in head kidney slices incubated either without (sham) or with ACTH (0.5 IU/ml) and sampled after 0.5, 1, 2, 3 and 4 h. All values represent mean ± S.E.M. (n = 4 fish); * significantly different from 0 h time control (paired t-test, $p<0.05$).

Figure 7. Ah receptor activation impairs in vitro ACTH-stimulated StAR (A), P450scc (B) and 11β-hydroxylase (C) transcript levels in rainbow trout. Head kidney tissue slices were exposed to control (ACTH), resveratrol (10^{-5} M RVT), β-naphthoflavone (10^{-6} M BNF) or RVT and BNF (RBNF) and stimulated with ACTH (0.5 IU/ml) and mRNA abundance levels were measured using real-time quantitative PCR; the data are shown as % unstimulated (in the absence of ACTH) control; all values represent mean + S.E.M (n = 4 fish); bars with different letters are statistically significant (One-way ANOVA, $p<0.05$).

Table 1. Oligonucleotide primers used in quantitative real-time PCR

Gene	Primers	Accession Number	Tm	Product size
CYP1A1	Sense 5'-GATGTCAGTGGCAGCTTTGA-3' Anti-sense 5'-TCCTGGTCATCATGGCTGTA-3'	U62796	60°C	100 bp
StAR protein	Sense 5'-CGCTGGCATCTCCTACA-3' Anti-sense 5'-GGGACTTCGTTAGTGTTCG-3'	AB047032	60°C	500 bp
P450scc	Sense 5'-GAGGAGGGTAGGAGCCA-3' Anti-sense 5'-CCTTGTGGGACTCTGGT-3'	S57305.1	58°C	500 bp
11β-hydroxylase	Sense 5'-ACCTCTTCCGCTTCGC-3' Anti-sense 5'-GGGTAGCGTGGGCAAGA-3'	AF179894	59°C	500 bp
β-actin	Sense 5'-AGAGCTACGAGCTGCCTGAC-3' Anti-sense 5'-GCAAGACTCCATACCGAGGA-3'	AF157514	49°C	100 bp

Fig.1.


A
CYP1A1
(56 kDa)
Liver CYP1A1 protein expression
(% control)
1200
1000
800
600
400
200
0
a
a
b
a
Control
RVT
BNF
RBNF
Treatments

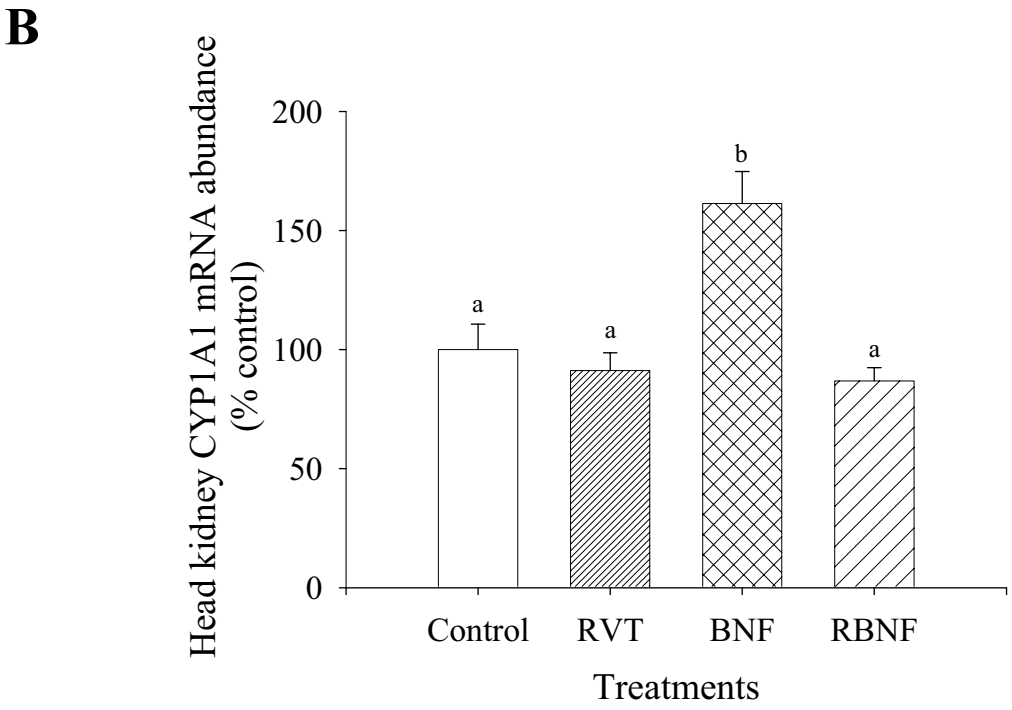

B
Head kidney CYP1A1 mRNA abundance
(% control)
200
150
100
50
0
a
a
b
a
Control
RVT
BNF
RBNF
Treatments

Fig.2.

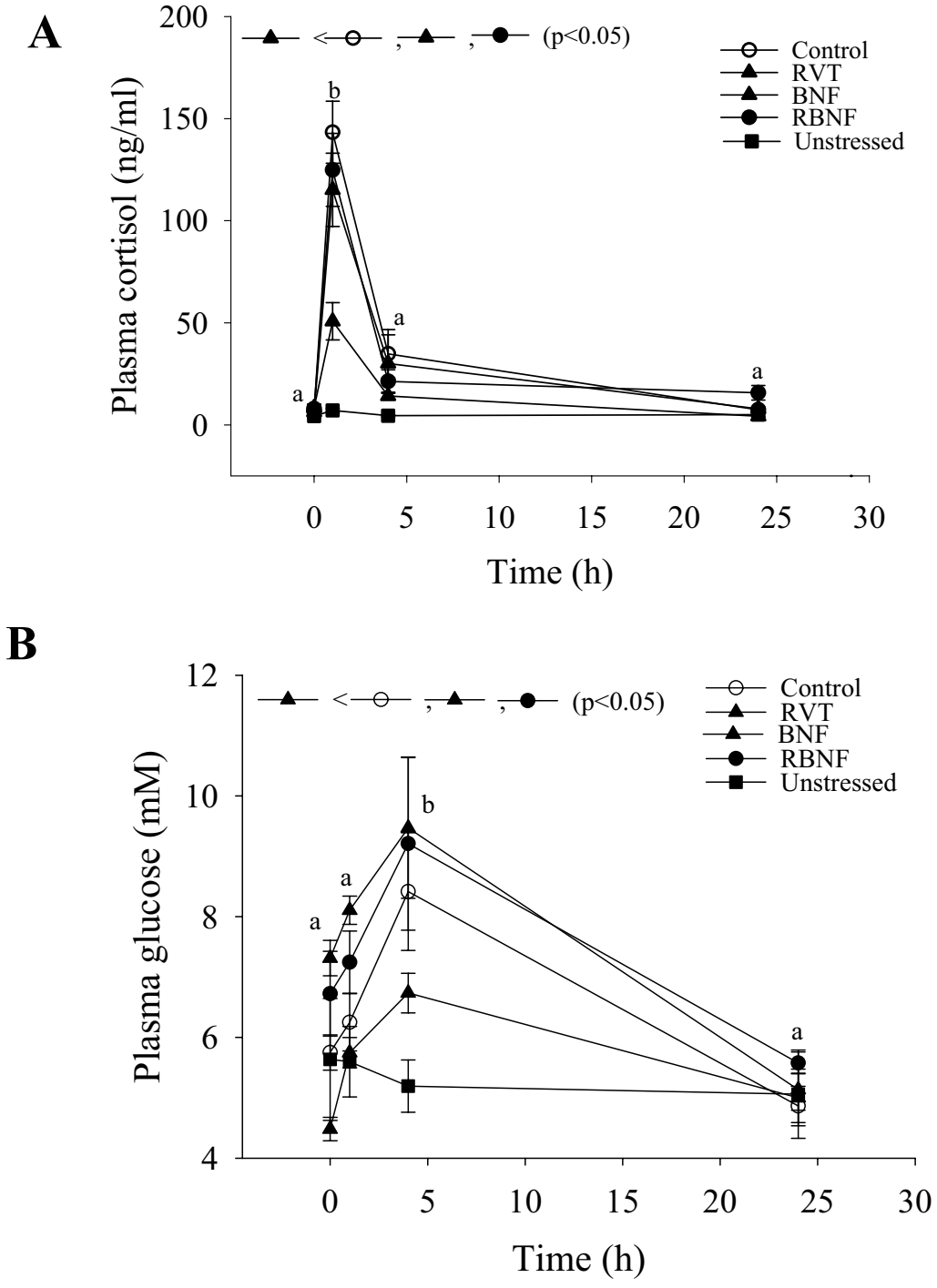

A
Plasma cortisol (ng/ml)
Time (h)
(p<0.05)
Control
RVT
BNF
RBNF
Unstressed
B
Plasma glucose (mM)
Time (h)
(p<0.05)
Control
RVT
BNF
RBNF
Unstressed

Fig.3

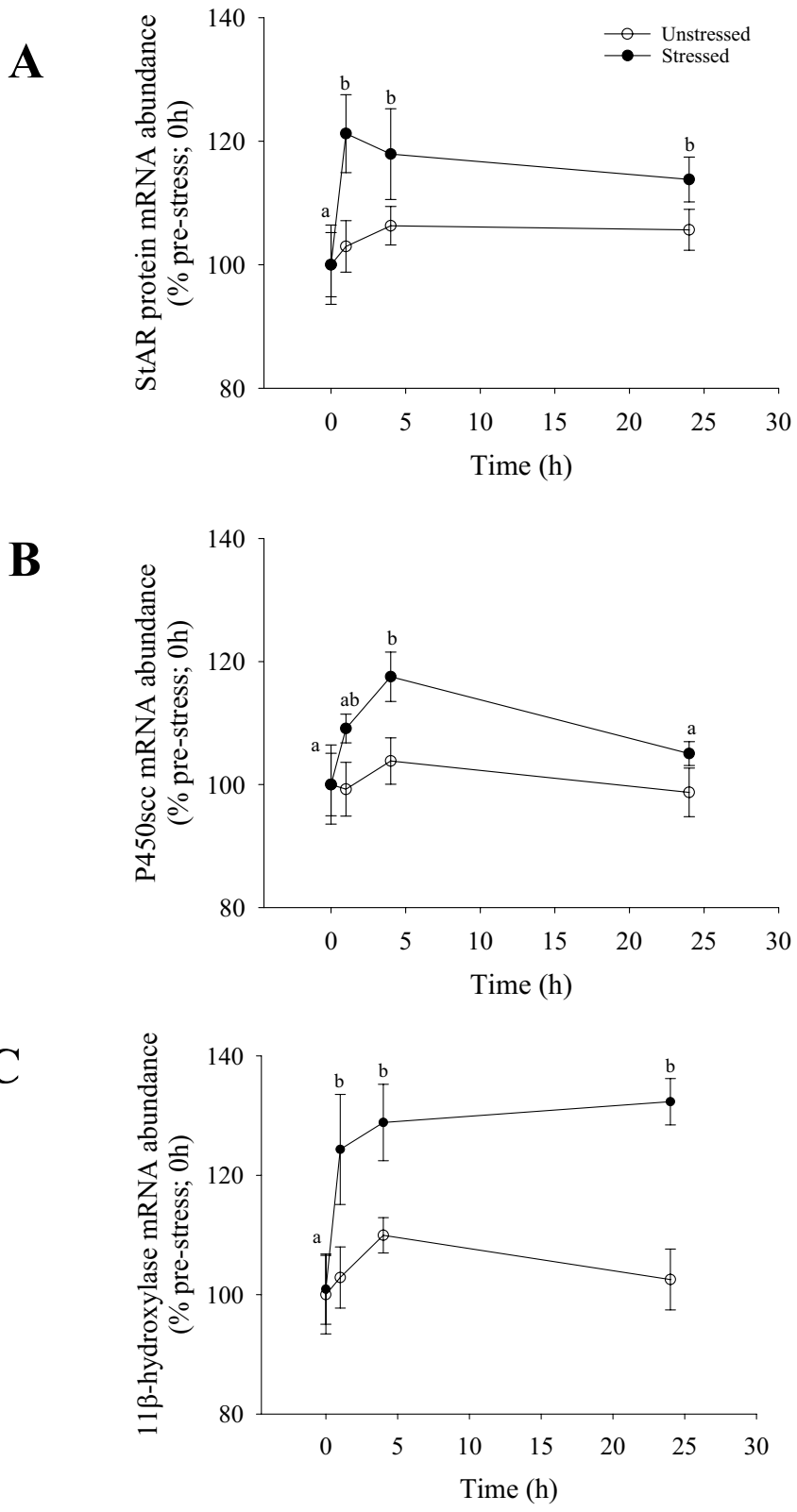

A
StAR protein mRNA abundance
(% pre-stress; 0h)
Unstressed
Stressed
140
120
100
80
0
5
10
15
20
25
30
Time (h)
a
b
b
b
B
P450scc mRNA abundance
(% pre-stress; 0h)
a
ab
b
a
C
11β-hydroxylase mRNA abundance
(% pre-stress; 0h)
a
b
b
b

Fig.4

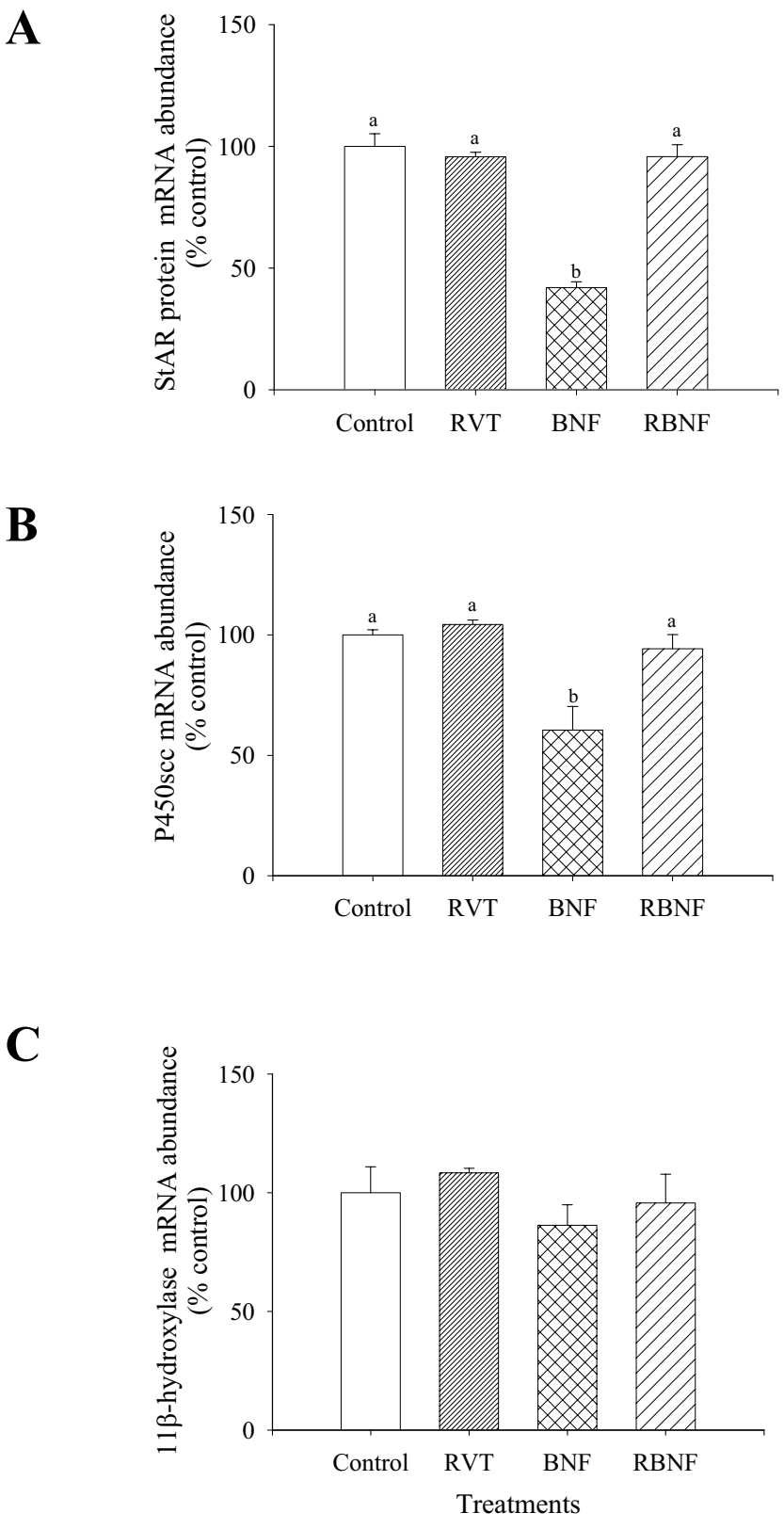

A
StAR protein mRNA abundance (% control)
150
100
50
0
a
a
b
a
Control
RVT
BNF
RBNF
B
P450scc mRNA abundance (% control)
150
100
50
0
a
a
b
a
Control
RVT
BNF
RBNF
C
11β-hydroxylase mRNA abundance (% control)
150
100
50
0
Control
RVT
BNF
RBNF
Treatments

Fig. 5

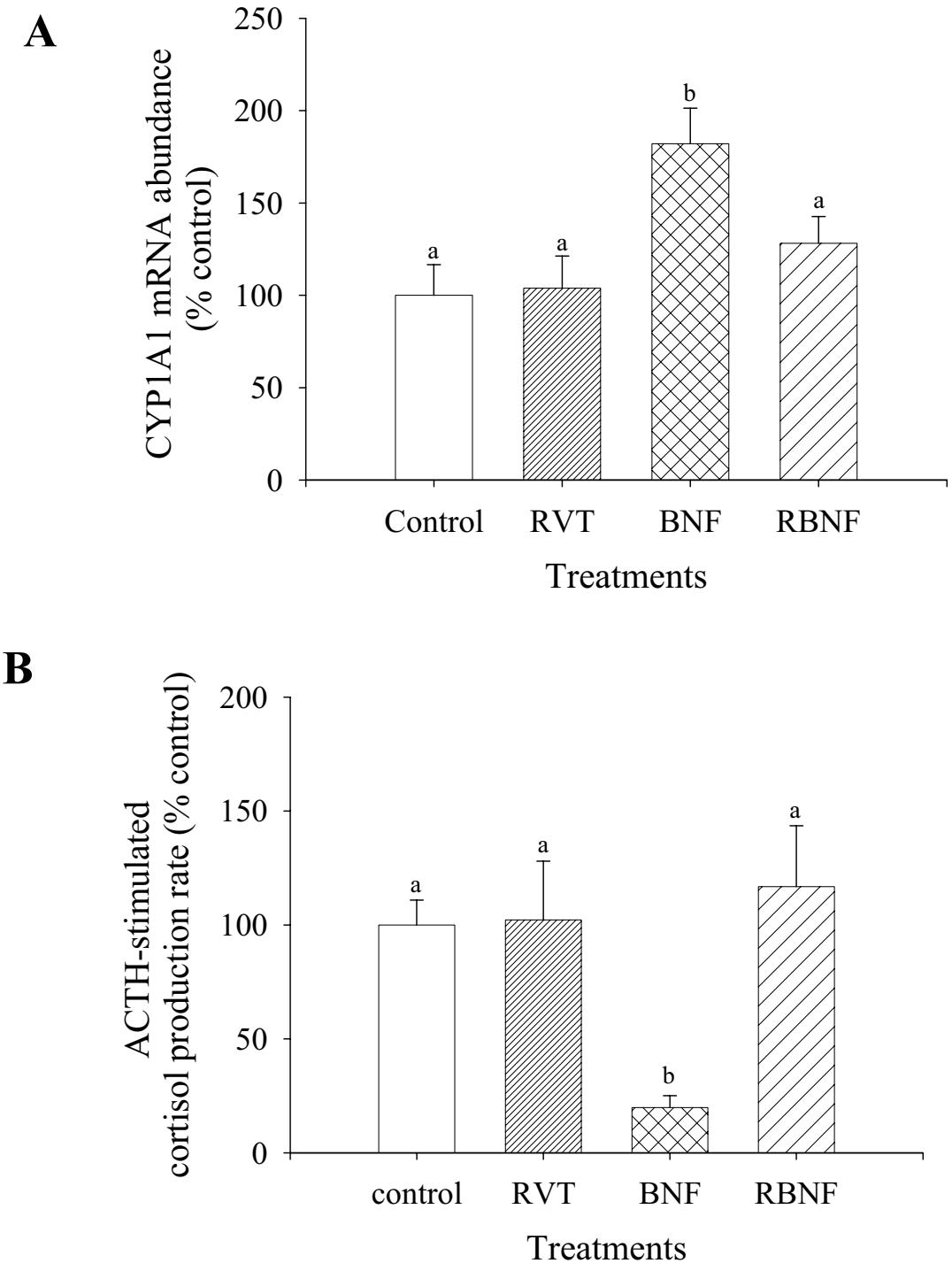

A
CYP1A1 mRNA abundance (% control)
250
200
150
100
50
0
a
a
b
a
Control
RVT
BNF
RBNF
Treatments
B
ACTH-stimulated cortisol production rate (% control)
200
150
100
50
0
a
a
b
a
control
RVT
BNF
RBNF
Treatments

Fig. 6

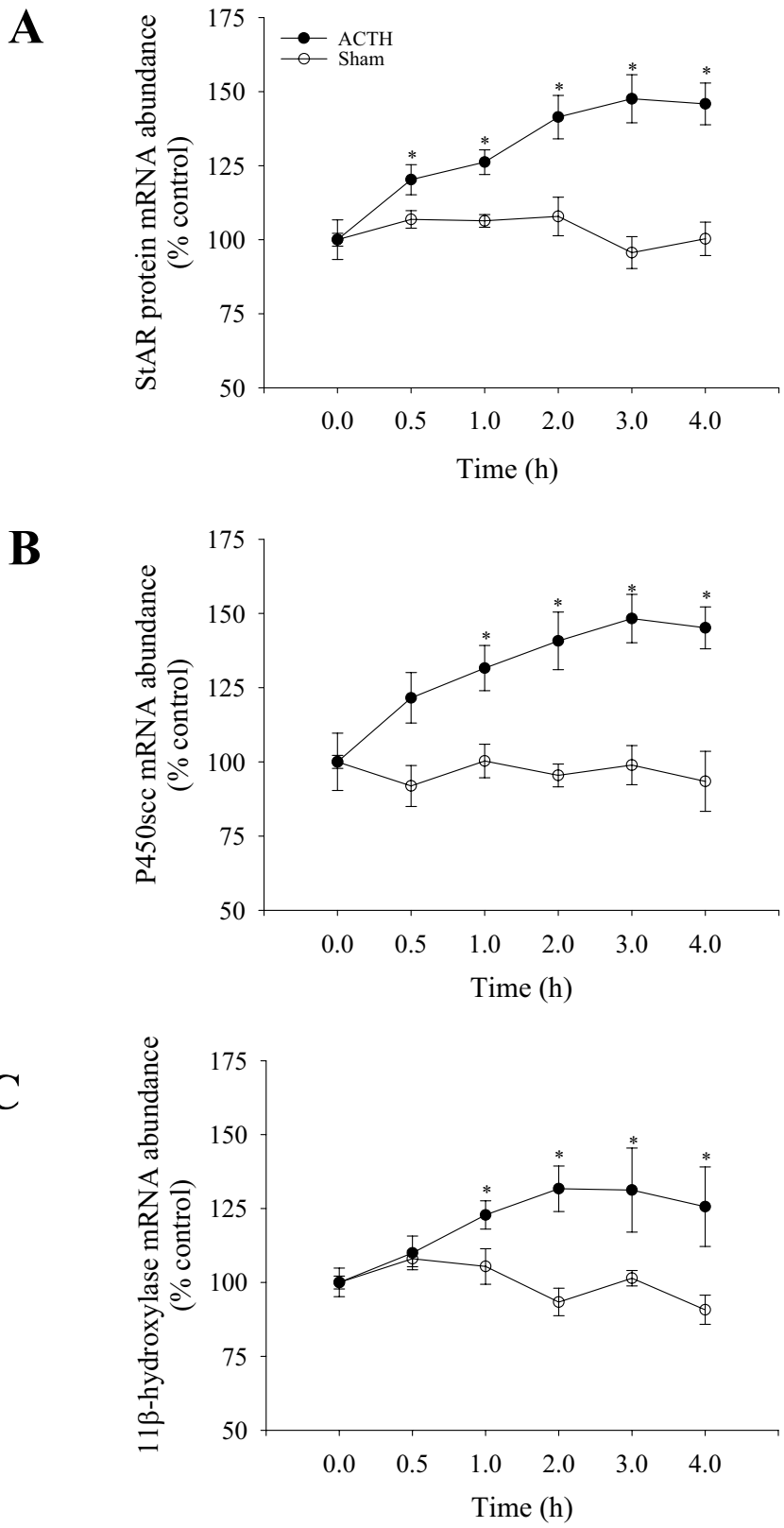
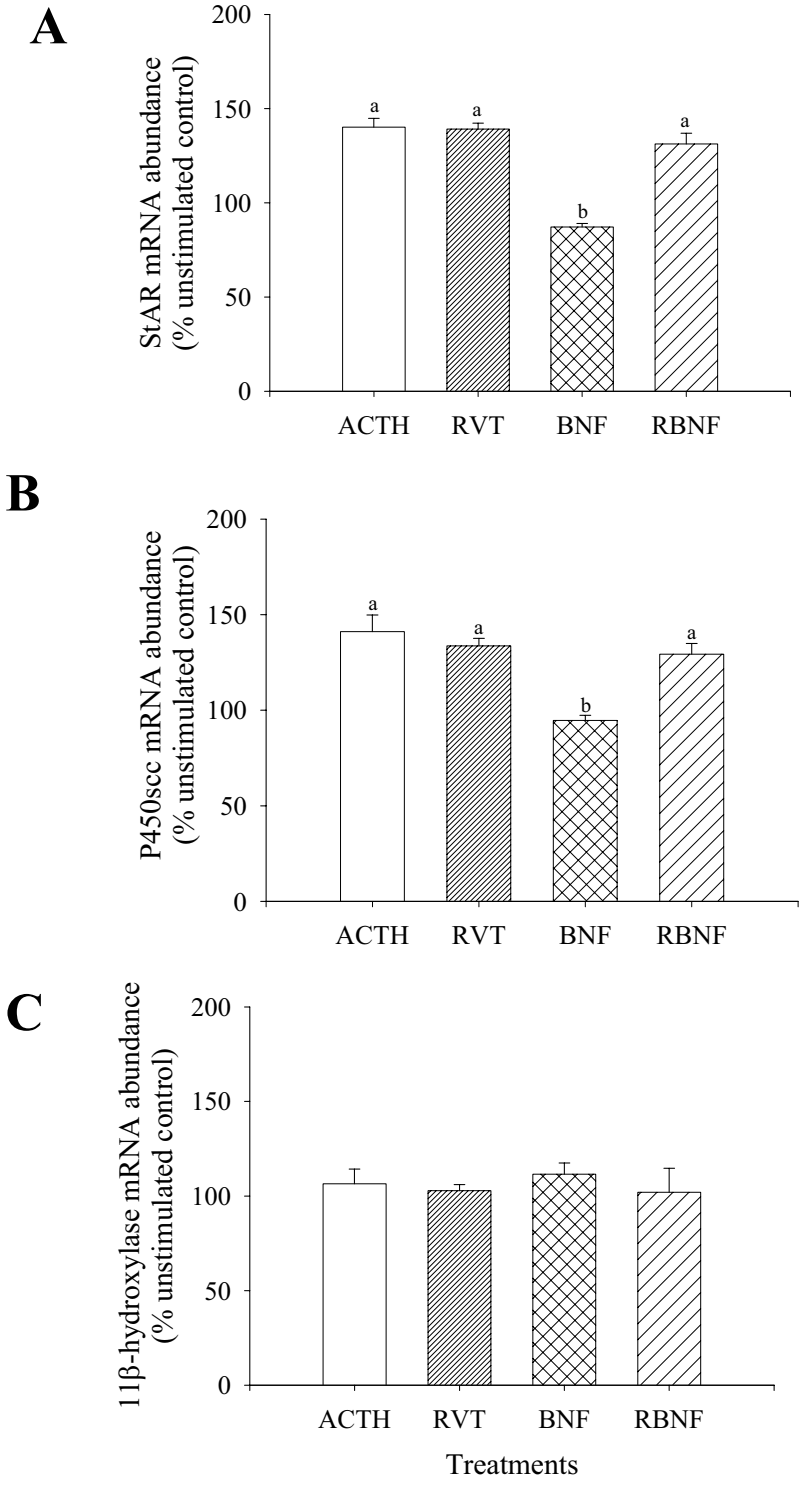

A
ACTH
Sham
StAR protein mRNA abundance (% control)
175
150
125
100
75
50
0.0
0.5
1.0
2.0
3.0
4.0
Time (h)
B
P450scc mRNA abundance (% control)
C
11β-hydroxylase mRNA abundance (% control)

Fig. 7.


A
StAR mRNA abundance
(% unstimulated control)
200
150
100
50
0
a
a
b
a
ACTH
RVT
BNF
RBNF
B
P450scc mRNA abundance
(% unstimulated control)
200
150
100
50
0
a
a
b
a
ACTH
RVT
BNF
RBNF
C
11β-hydroxylase mRNA abundance
(% unstimulated control)
200
150
100
50
0
ACTH
RVT
BNF
RBNF
Treatments